JHeimbach LLC

April 13, 2020

Susan J. Carlson, Ph.D., Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, Advantage Enzymes Technologies, Ltd., through me as its agent, hereby provide notice of a claim that the addition of *Bacillus coagulans* strain LBSC to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Advantage Enzymes Technologies has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the conclusion from the members of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the signed statement of the Expert Panel.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5543 or jh@jheimbach.com.

Sincerely

(b) (6)

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

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GRAS NOTIFICATION

Bacillus coagulans LBSC (DSM 17654)

Advanced Enzyme Technologies Ltd.
5th Floor, 'A' wing, Sun Magnetica,
L.I.C. Service Road, Louiswadi,
Thane (W) – 400 064, INDIA

Edited by
JHeimbach LLC
Port Royal Virginia USA

April 2020

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Part 1. Signed Statements and Certifications

1.1 GRAS Notice Submission

Advanced Enzymes Technologies Ltd. submits this GRAS notice in accordance with 21 CFR part 170, subpart E.

1.2 Name and Address of Notifier

APPLICANT

Name:	Advanced Enzyme Technologies Ltd.
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MANUFACTURER

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PERSON RESPONSIBLE FOR THE DOSSIER

Name:	Dr. Anil Kumar Gupta, VP – Research & Development Advanced Enzyme Technologies Ltd.
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US AGENT

Name:	James T. Heimbach, Ph.D., F.A.C.N. JHeimbach LLC
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Postal code and City:	Port Royal, Virginia 22535
County:	USA
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1.3 Name of Notified Microorganism

Bacillus coagulans strain LBSC. 'LBSC' is the designation of the proprietary *Bacillus coagulans* strain of Advanced Enzyme Technologies Ltd. The strain was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under strain designation DSM 17654.

The product *Bacillus coagulans* LBSC (DSM 17654) is a spore preparation containing no viable vegetative cells. Commercial preparations are known as SEBiotic, ProFood BC, and BioSEB BC.

In this GRAS notice, *Bacillus coagulans* strain LBSC is referred to by shorter names such as *Bacillus coagulans* LBSC, *B. coagulans* LBSC, or DSM 17654. In the literature, the species is occasionally denominated by the obsolete (and scientifically incorrect) names *Lactobacillus sporogenes* or "lactic acid Bacillus."

1.4 Intended Conditions of Use

Bacillus coagulans LBSC is intended to be used in the following food categories:

Baked goods and baking mixes, breakfast cereals, beverages and beverage bases, coffee and tea; milk and milk products, dairy product analogs, fruit juices, condiments and relishes, confections and frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, gelatins, jams and jellies, puddings and fillings, alcoholic beverages, grain products and pastas, hard candy, soft candy, chewing gum, extracts, and flavorings, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, processed fruits, processed vegetables and vegetable juices, snack foods, soups and soup mixes, sugar and sweet sauces, toppings, and syrups at a maximum level of approximately 1×10^8 to 2×10^9 colony forming units (cfu)/serving.

Based upon the estimated number of servings of food consumed per day in the US and the highest intended addition level of *Bacillus coagulans* per serving, the estimated daily intake (EDI) of the strain is 36.4×10^9 cfu/day. (This EDI, of course, would be reached only if all target foods indeed contained *B. coagulans* at the maximum addition level.) The intended use of *B. coagulans* strain LBSC is identical to the use of *Bacillus coagulans* strains previously determined to be GRAS [GRN 399 (2011); GRN 526 (2014); GRN 597 (2015); GRN 601 (2015); GRN 691 (2017)] and therefore would provide an alternate source of the microorganism in the spore preparation added to these foods but would not result in any change in exposure to the species.

B. coagulans LBSC is not intended for use in foods that are targeted toward infants, such as infant formulas or foods formulated for infants, nor in meat and poultry products that come under USDA jurisdiction.

1.5 Statutory Basis for GRAS Status

Advanced Enzyme Technologies Ltd., has determined that the intended use of *Bacillus coagulans* LBSC is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

1.6 Premarket Exempt Status

Since Advanced Enzyme Technologies Ltd. has determined that the intended use of *Bacillus coagulans* LBSC is GRAS, the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data Availability

Advanced Enzyme Technologies Ltd. agrees to make the data and information that are the basis for the determination of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzyme Technologies Ltd. to FDA either in electronic format or on paper or reviewed during customary business hours at the home office of JHeimbach LLC, located at 923 Water Street, Port Royal VA 22535.

1.8 FOIA Statement

None of the data and information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.9 Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bacillus coagulans* strain LBSC.

1.10 FSIS Statement

Not applicable.

1.11 Name/Position of Notifier

(b) (6)

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Advanced Enzymes Technologies Ltd.

Part 2. Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity/ Identification

The substance that is covered in this GRAS notification is a preparation of *B. coagulans* LBSC spores, which contains no viable revetative cells. It is a member of a subgroup of *Bacillus* spp. The diluents used in the manufacturing of *Bacillus coagulans* LBSC are approved as either food additives or GRAS substances.

2.1.1 *SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

Name of the food ingredient: *Bacillus coagulans* strain LBSC (DSM 17654)

Synonyms: *Bacillus coagulans* strain LBSC / *Bacillus coagulans* (strain LBSC) / *B. coagulans* LBSC / Lactic Acid Bacillus (obsolete and scientifically incorrect) / *Lactobacillus sporogenes* (obsolete and scientifically incorrect)

Taxonomy:
Kingdom: Bacteria
Phylum: Firmicutes (Gram positive spore forming bacteria)
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus
Species: coagulans

B.W. Hammer at the Iowa Agricultural Experiment Station first described *Bacillus coagulans* in 1915 as a causative agent for coagulation of condensed milk (Hammer, 1915). In 1935, *Bacillus coagulans* was identified as *Lactobacillus sporogenes* in the Fifth Edition of Bergey's Manual since it shared characteristics of both genera *Lactobacillus* and *Bacillus*. In 1957, it was finally transferred to the genus *Bacillus* in the seventh edition of Bergey's (Reed et al., 1957). The bacterium is still occasionally erroneously referred to as *Lactobacillus sporogenes*.

2.1.2 *DESCRIPTION/SOURCE INFORMATION AND GENOTYPIC, PHENOTYPIC CHARACTERIZATION OF THE ORGANISM*

B. coagulans LBSC, originally isolated from soil, is a nonpathogenic, non-toxicogenic, naturally encapsulated spore-forming bacterium, light brown to brown colored powder with characteristic odor having total viable count not less than 150 billion cfu/g. *B. coagulans* LBSC is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ) Germany with deposit number DSM 17654.

2.1.2.1 Genotypic Characterization

Genotypic characterization of *B coagulans* LBSC was carried out using the following tests: 16S rRNA and gyrB gene, DNA-DNA hybridization, genomic sequencing, determination of mole G+C% content. The *B. coagulans* LBSC genome is sequenced and a genome-based safety assessment was published (Saroj and Gupta 2020). The parameters described below were assessed to establish the safety of the intended use of *B. coagulans* LBSC.

a) 16S rRNA and gyrB gene

B. coagulans LBSC was identified using gyrB and 16S rRNA genes as phylogenetic markers. Closely related *Bacillus* species cannot be distinguished by 16S rRNA sequence analysis alone. Therefore, the gyrB gene, which encodes the subunit B protein of DNA gyrase, was selected as an additional phylogenetic marker. The 16S rRNA sequence and gyrB gene sequences showed 99% homology to *Bacillus coagulans* type strains and the gyrB sequence of *B. coagulans* LBSC showed 96% homology to *Bacillus coagulans* ATCC 7050.

b) DNA-DNA hybridization

Whole genome homology was evaluated by DNA-DNA hybridization studies which indicated 91.2% homology between *B. coagulans* strain LBSC (DSM 17654) and *B. coagulans* ATCC 7050. The results align with the requirement of ≥70% similarity, the main criterion for assigning two strains to the same species (Meier-Kolthoff et al., 2014).

c) Genomic Sequencing

Hybrid assembly was performed using SPAdes software (Bankevich et al., 2012) between error-corrected nanopore reads and Illumina processed reads, which was followed by reference-guided scaffolding and super-scaffolding using Ragout software (Kolmogorov et al., 2014) and Gap-Closer software (Simpson and Durbin, 2012). In this case, *B. coagulans* HM-08 strain was used as a reference. The final genome assembly was 3,635,902 bp in size with 46.30% G+C content. The whole-genome shotgun project was deposited in NCBI/GenBank under the accession number CP022701.

The assembled genome of *B. coagulans* LBSC was compared with three other *B. coagulans* strains, HM-08, ATCC 7050, and S-lac. An overall genome BLAST (Altschul et al., 1990) indicates the homology between the assembled genome with respect to HM-08, ATCC 7050 and S-lac to be 99%, 96%, and 99% respectively.

d) Determination of mol G+C%

The genomic DNA G+C content, defined as the proportion of guanines and cytosines within the overall number of nucleotides in the genome, is one of the features in taxonomic descriptions of microorganisms (Meier-Kolthoff et al., 2014). The mol % G+Cs estimated for *B. coagulans* strain LBSC (DSM 17654) in the present study by conventional methods and based on the full genome sequence were 47.2% and 46.30%, respectively, which are in agreement to the values reported for *B. coagulans* (44 to 50%) by Sudha et al. (2010).

e) Safety assessment in relation to antibiotic resistance genes

A homology search between the assembled genome of *B. coagulans* strain LBSC and antibiotic resistance genes was performed using the Comprehensive Antibiotic Resistance Database (CARD). In this case, BLASTX was used with the criteria similarity >30%, coverage >70%, and e-value < 1e-02 for the identification of significant hits. Through the above analysis, 144 putative antibiotic resistance genes were identified, which belonged to the following functions: cell wall/membrane/envelope biogenesis (21); defense mechanism (27); carbohydrate, lipid, amino acid, nucleotide coenzyme, and inorganic ion transport and metabolism (41); signal transduction mechanism transcription, DNA binding transcription regulator (25); general function and prediction (22); translation, ribosomal structure, and biogenesis (4); replication, recombination, and repair (2); vancomycin resistance gene and posttranslational modification, protein turnover,

chaperones (1). The absence of mobile elements in the flanking regions of the above-mentioned antibiotic resistance genes determined using ISfinder web-based software (Siguier et al., 2006) and ACLAME database (Leplae et al., 2010), indicates high stability of these regions. This analyses did not reveal any active pathways for antibiotic resistance and showed that *B. coagulans* LBSC is safe from horizontal antibiotic resistance gene transfer.

To confirm the above analysis, *B. coagulans* strain LBSC was tested as per CLSI guidelines for its sensitivity/resistance against nine antibiotics, ampicillin, vancomycin, gentamicin, kanamycin, streptomycin , erythromycin, clindamycin, tetracycline and chloramphenicol. *B. coagulans* strain LBSC was sensitive to all tested antibiotics. The minimum inhibitory concentration (MIC) breakpoint values observed for *B. coagulans* strain LBSC were below or equal to the break point values described by EFSA (2012). (See also Section 1.12.3.)

f) Analyses of risk associated with virulence factor genes

Virulence factor genes/proteins were downloaded from the Virulence Factor Database (VFDB). The total number of sequences in the core database was 412. A homology search between the assembled genome of *B. coagulans* strain LBSC and virulence factor proteins was performed using BLASTX (criteria: similarity >30%, coverage >70%, and e-value < 1e-02) to identify significant hits. A total of 76 virulence factor proteins were found to have significant homology with the assembled genome. All these genes were non-classical virulence factor genes and their determinants were related to cell wall/membrane/envelope biogenesis (28); signal transduction mechanism (14); carbohydrate, inorganic ion, coenzyme, and amino acid transport and metabolism (8); cell cycle control, cell division, chromosome partitioning (7); transcription (4); and general function (14).

The absence of mobile elements in the flanking regions of the above-mentioned virulence factor genes/proteins determined using ISfinder web-based software and ACLAME database indicates high stability of these regions. The results shows no safety concern in *B. coagulans* LBSC with regard to virulence factors.

g) Identification of biogenic amine producing genes

Protein sequences of the biogenic amine producing genes were downloaded from the Uniprot database. BLASTX was performed between the assembled genome and biogenic amine producing proteins. It was observed that only 3 sequences of the genomic region showed significant hits against biogenic amine producing proteins, carboxynorspermidine decarboxylase, agmatinase, and arginine decarboxylase. Further, the presence of these 3 proteins was also validated by NCBI prokaryotic genome annotation. The phenotypic assay did not detect the production of biogenic amines in the conditions tested, suggesting that the genes are non-functional and the strain does not produce biogenic amines.

h) Identification of mobile elements in assembled genome

Mobile elements are DNA sequences that can move around the genome by changing their number of copies or simply by changing their location, often affecting the activity of nearby genes. In the current study, mobile elements were predicted by using ISfinder software. A total of 31 insertion sites (IS element regions) were identified in the assembled genome. A BLASTN was performed between the nucleotide sequences (plasmids, viruses, and prophages downloaded from the ACLAME database) and the assembled genome. There were 34 regions that have significant hits against the mobile-element nucleotide sequences downloaded from the ACLAME database.

None of the regions of concern—antibiotic resistance genes, virulence factor genes, and biogenic amine producing genes—were observed in the vicinity of the predicted mobile elements in the assembled genome, thus ensuring the stability of the genome and consistant safe use of the strain.

i) Analyses of toxin genes

Genes involved in the production of lipopeptides and enterotoxin were analyzed in the assembled genome sequence of *B. coagulans* strain LBSC based on percentage identity ≥30%. Genes encoding for surfactins, cyclic lipopeptides, fengycin (Salvetti et al., 2016), and lychenisin and enterotoxin genes (Nhe, enterotoxin K and T, emetic toxin gene) were not present in the assembled genome, suggesting that *B. coagulans* LBSC does not produce these toxins and is safe for human consumption.

j) Identification of CRISPR associated regions in assembled genome

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences were screened in the assembled genome of *B. coagulans* strain LBSC using CRISPRFinder (Zhang et al., 2017). One confirmed CRISPR motif followed by 34 different spacer sequences was identified from the assembled genome. The presence of a CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to entry of foreign DNA elements.

Conclusion

The *de novo* assembled genome of *B. coagulans* strain LBSC, generated without gaps, resulted in a single scaffold. There are no significant mobile elements identified with respect to the loci which have significant homology against antibiotic resistance genes, virulence factor genes, biogenic amine producing genes, or enterotoxin genes. The presence of a CRISPR sequence in the assembled genome indicates an advantage in promoting genome stability by acting as a barrier to the entry of foreign DNA elements. In conclusion, *B. coagulans* strain LBSC does not contain any sequences/genes in the genome that are risk associated, thus confirming the safety of the strain through the genome-based approach.

2.1.2.2 Phenotypic and Biochemical Characterization

B. coagulans LBSC is an endospore forming, rod-shaped, Gram-positive, facultative anaerobic, catalase-positive, slightly acidophilic, thermotolerant, and aerobic to microaerophilic bacterium. Its cell size ranges from approximately 0.7 µm in width and 1.7 µm to 5.2 µm in length. Cells are arranged singly or in short chains. After 2 days of incubation on trypticase soy agar at 40°C, colonies are <1 to 3 mm in diameter, white, convex, with entire margins and smooth surfaces; they become cream-colored with age. Spores are ellipsoidal but sometimes appear spherical; they lie sub-terminally and occasionally paracentrally or terminally in unswollen sporangia.

B. coagulans LBSC is positive for catalase, oxidase, gelatinase, protease (casein), and amylase enzymes. The strain also showed a positive result in the Methyl Red test and showed a weakly positive result for the Voges-Proskauer test. In the TSI test, the strain showed no gas or hydrogen sulfide production but showed yellow (acidic) butt and slant. The strain showed a negative test result for urease, nitrate reductase, tryptophanase, and citrate utilization. *B. coagulans* LBSC was able to ferment the following carbohydrates: D-glucose, sucrose, lactose, maltose, starch, dextrin, glycerol, mannitol, rhamnose (weakly positive), D-fructose, D-galactose, iInulin (weakly positive), D-mannose, D-sorbitol, and D-trehalose and was unable to ferment xylose or L-arabinose (Table 1).

The results of biochemical tests of *B. coagulans* LBSC were comparable to the reference strain of *Bacillus coagulans* ATCC 7050; the data provided by Logan et al. (2015) and *Bergey's Manual of Systematics of Archaea and Bacteria* are provided in Table 1. The strain was characterized as a member of the genus of *Bacillus* and species *coagulans*.

Table 1. Results of Morphological and Biochemical Tests

Test	Results	
	***Bacillus coagulans* LBSC**	***Bacillus coagulans* ATCC7050**
Colony Characteristics	Colonies white, convex with entire margins and smooth surfaces, dark centered, cream-colored	Colonies white, convex with entire margins and smooth surfaces; become cream-colored with age
Gram Staining	Gram positive	Gram positive
Cell Morphology	Cells motile, rod shaped, with peritrichous flagella	Cells motile, rod shaped, with peritrichous flagella
Size	Approximately 0.7 µm in width and 1.7 µm-5.2 µm in length	0.5 µm-0.7 µm in width and 1.6 µm-7.1 µm in length
Arrangement	Mostly single cells, less in short chains	Single cells or in short chains
Catalase Test	Positive	Positive
Oxidase Test	Positive	Positive
Nitrate Reduction Test	Negative	Negative
Endospore stain	Spores ellipsoidal but sometimes appear spherical; lie subterminally and occasionally paracentrally or terminally in unswollen sporangia	Spores ellipsoidal but sometimes appear spherical; lie subterminally and occasionally paracentrally or terminally in unswollen sporangia
Indole Test	Negative	Negative
Methyl Red Test	Positive	Positive
Voges-Proskauer Test	Weakly positive	Weakly positive
Citrate Utilization Test	Negative	Negative
Urease Test	Negative	Negative
Triple Sugar Iron (H_2S) Test	No production of hydrogen sulphide, No gas production, Yellow Slant and Butt	No production of hydrogen sulfide, No gas production, Yellow Slant and Butt
Gelatin hydrolysis Test	Weakly positive	Variable
Casein hydrolysis Test	Positive	Positive
Starch hydrolysis Test	Positive	Positive
Sugar Fermentation Tests		
D-Glucose	Acid produced, No gas produced	Acid produced, No gas produced
Sucrose	Acid produced, No gas produced	Acid produced, No gas produced
Lactose	Acid produced, No gas produced	Acid produced, No gas produced
Maltose	Acid produced, No gas produced	Acid produced, No gas produced
Starch	Acid produced, No gas produced	Acid produced, No gas produced
Dextrin	Acid produced, No gas produced	Acid produced, No gas produced
Glycerol	Acid produced, No gas produced	Acid produced, No gas produced
Mannitol	Acid produced, No gas produced	Acid produced, No gas produced
Xylose	Negative for both acid and gas production	Negative for both acid and gas production
Rhamnose	Weakly positive	Weakly positive
D-Fructose	Acid produced, No gas produced	Acid produced, No gas produced
D-Galactose	Acid produced, No gas produced	Acid produced, No gas produced
D-Mannose	Acid produced, No gas produced	Acid produced, No gas produced
L-Arabinose	Negative for both acid and gas production	Negative for both acid and gas production
Inulin	Weakly positive	Weakly positive
D-Sorbitol	Acid produced, No gas produced	Acid produced, No gas produced
D-Trehalose	Acid produced, No gas produced	Acid produced, No gas produced

2.1.3 *ANTIBIOTIC RESISTANCE (SUSCEPTIBILITY)*

Three batches of the *B. coagulans* LBSC strain were assessed for antibiotic susceptibility following CLSI (2012) guidelines as recommended by EFSA (2012).

Agar double dilution assays were used to evaluate the antibiotic susceptibility of *B. coagulans* LBSC against eight antibiotics and to determine the minimum inhibitory concentration (MIC: the lowest concentration of the antibiotic that inhibits bacterial growth). Antibiotics tested included clindamycin, chloramphenicol, gentamicin, tetracycline, streptomycin, kanamycin, vancomycin, and erythromycin, with the results shown in Table 2.

Table 2. Antibiotic Susceptibility of *Bacillus coagulans* LBSC

Antibiotic	*Staphylococcus aureus* ATCC 29213			*Bacillus coagulans* LBSC		
	MIC range[1] (µg/ml)	MIC (µg/ml)	Interpre-tation	MIC break-point[4] (µg/ml)	MIC (µg/ml)	Interpre-tation
Clindamycin	0.06 – 0.25	0.12	S[3]	4	1	S
Chloramphenicol	2 – 16	16	S	8	8	S
Ampicillin	0.5 – 2	2	S	NR[5]	NR	NR
Gentamicin	0.12 – 1	1	S	4	2	S
Tetracycline	0.12 – 1	0.25	S	8	1	S
Streptomycin	NA[2]	NA	NA	8	8	S
Kanamycin	1 – 4	4	S	8	8	S
Vancomycin	0.5 – 2	2	S	4	2	S
Erythromycin	0.25 - 1	1	S	4	1	S

1. Source: CLSI, 2012
2. NA = not available in CLSI (2012)
3. S = susceptible
4. Source: EFSA, 2012
5. NR = not required (EFSA, 2012)

B. coagulans LBSC was found to be sensitive to all eight tested antibiotics. MIC values observed were in accordance with the epidemiologic cut-off (ECOFF) values as described by EFSA (2012).

The antibiotic sensitivity profile of *B. coagulans* LBSC was also checked by the disk-diffusion method. The antibiogram profile was compared with the control strain, *Bacillus coagulans* ATCC 7050 (GRN 691). Antibiotics tested included amikacin, amoxicillin with clavulanic acid, ampicillin, bacitracin, cefaclor, cefamandole, cefazolin, cefoperazone, cefoxitin, ceftizoxime, cefuroxime, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, methicillin, nitrofurantoin, norfloxacin, colistin, nalidixic acid, doxycycline, penicillin, ticarcillin/clavulanic acid, streptomycin, novobiocin, polymyxin, ciprofloxacin, rifampicin, and tetracycline at the given concentrations.

Both *B. coagulans* LBSC and the control were sensitive to the aforementioned antibiotics. It was also observed that both *B. coagulans* LBSC and the control were resistant to aztreonam, cefonicid, and ceftazidime at the tested antibiotic concentrations.

2.1.4 *VIRULENCE ACTIVITY*

A test for cytotoxicity using Vero cells was performed to demonstrate that *B. coagulans* LBSC is free from toxigenic potential (EFSA 2014).

The test is based on the principle that the DNA intercalating agent propidium iodide will stain DNA of cells having leaky cell membranes, thereby enhancing the resulting intracellular fluorescent signal. The DNA of intact cells would not show any uptake of propidium iodide, resulting in basal level, negligible fluorescence. The study showed that the sample of *B. coagulans* LBSC did not elicit cytotoxicity on Vero cells (Table 3).

Table 3. Test for Detection of Cytotoxicity Using Vero cells

Test Article	Fluorescence Units in Live Cells	T/C* (%)
Background	0	0
Positive control	820	4.82
Negative control	17014	100.00
B. coagulans LBSC – 10 µl	12897	75.80
B. coagulans LBSC – 50 µl	10796	63.45
B. coagulans LBSC – 100 µl	10096	59.34
*T/C% = survival ratio of treated to control cells		

T/C% values for samples of *B. coagulans* LBSC were more than 30%, indicating that the sample did not have any cytotoxic effect *in vitro* at 10-100 µl sample volume for the 2-hour incubation period.

2.1.5 *ANTIMICROBIAL ACTIVITY*

B. coagulans LBSC was evaluated for its antimicrobial activity following CLSI (2012) guidelines as recommended by EFSA (2012) [*Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212] and the United States Pharmacopoeia (USP, 2008) [*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Staphylococcus epidermis* ATCC 12228]. *B. coagulans* LBSC showed an absence of antimicrobial activity against the select test micro-organisms.

2.1.6 *ACID AND BILE SALT TOLERANCE*

The spore preparation of *B. coagulans* LBSC was tested for its ability to survive under different simulated gastrointestinal conditions through an *in vitro* study.

After 24 hours of exposure, *B. coagulans* LBSC was stable in simulated saliva (95.58%), Fed State Simulated Gastric Fluid (70.18%), simulated intestinal fluid (71.30%), and simulated colonic fluid (96.55%), slightly reduced in simulated gastric juice (less than 0.6 log cycle; 61%) at 60 minutes and maintained a significant viable activity up to stomach transit time, log10 cfu/ml, 8.36 (21%) after 90 minutes.

In vitro studies revealed *B. coagulans* LBSC was stable and maintained its survivability under different simulated gastrointestinal conditions.

2.1.7 *ENTEROTOXINS*

B. coagulans LBSC was checked for enterotoxins by Duopath® Cereus Enterotoxins test kit, Merck. The organism was concluded to be negative for non-hemolytic enterotoxins and hemolysin BL enterotoxins.

Conclusion

B. coagulans LBSC strain has been thoroughly analysed for risk-associated factors following genome based analyses and phenotypic/biochemical studies. Various studies/analyses carried out on this strain showed no safety concern and concluded that the strain is safe for human consumption.

2.2 Manufacturing Process

2.2.1 *OVERVIEW*

B. coagulans LBSC is produced as spores by batch and fed-batch type fermentation. Fermentation is in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001, ISO 22000 and GMP certified.

B. coagulans LBSC is produced by fermentation. Fermentation is a well-known process that occurs in food and has been used for the cultivation of microorganisms for decades. Liquid state or submerged fermentation is used to produce the *B. coagulans* LBSC. The typical fermentation batch size ranges from 100 L to 50,000 L, preferably 14,000 to 20,000 L. The frequency of production is planned depending on the market demand.

As shown below, the key steps for production of *B. coagulans* LBSC are fermentation, recovery, formulation, and packaging. The process is illustrated in Figure 1.

2.2.2 *FERMENTATION*

2.2.2.1 Raw materials

Materials used in the fermentation process (inoculum, seed, and main fermentation) are all food-grade substances approved for this use. There are no ingredients based on milk, soy, or any of the top eight allergens.

- Potable water
- A carbon source
- A nitrogen source
- Salts
- Vitamins (as a part of complex fermentation materials)
- pH adjustment agents
- Foam control agent (at ≤0.1%)

2.2.2.2 Inoculum (Seed)

A suspension of a pure culture of *B. coagulans* LBSC is aseptically transferred to an inoculum flask containing fermentation medium.

The culture is grown in the flask under optimum conditions in order to obtain a sufficient amount of biomass, which can subsequently be used as inoculum for the seed fermentation.

2.2.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium. When a sufficient amount of biomass has developed (typically up to 17 hours), the content of the seed fermenter is used for inoculation of the main fermentation.

2.2.2.4 Main fermentation

During the main fermentation, the growth (cell-mass) of *B. coagulans* LBSC takes place and the vegetative cells later converted to spores during late growth/stationary phase.

The fermentation in the main fermenter is operated as a batch and fed-batch fermentation. First, the content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium. The fermentation process is continued for a predetermined time or until

laboratory test data show that the desired biomass production has been obtained or that the rate of biomass production has decreased below a predetermined production rate. When the desired spore count is reached, the fermentation is complete. At this time the temperature is raised to 75ºC and held for 30 minutes, which assures that any vegetative cells are killed and the only viable organisms in the final product are spores.

2.2.3 *RECOVERY*

The purpose of the recovery process is to separate the *B. coagulans* LBSC spores from the fermentation media, concentrate the spores, and prepare dried powdered biomass.

The vegetative cells of *B. coagulans* LBSC are converted to spores at the end of fermentation and are suspended in the fermentation media. During recovery, spores are separated from fermentation medium.

The steps of recovery include:

- Primary separation of spores (biomass) from the soluble media components
- Washing of concentrated spores (biomass)
- Spray drying

2.2.3.1 Primary Separation

The fermentation broth is passed through a high-speed centrifuge to separate the spores (biomass) from the soluble media components along with water. The spore biomass is collected as a thick slurry and subjected to further processing. Temperature and pH are controlled during this step.

2.2.3.2 Washing

Sterilized and demineralized water is added to the collected biomass slurry. Slurry is again passed through high-speed centrifuge and the washed biomass is collected. Temperature and pH are controlled during this step.

2.2.3.3 Spray Drying

The concentrated biomass suspension is spray-dried in presence of approved food-grade stabilizers (e.g., maltodextrin) to obtain the unformulated concentrate.

2.2.4 *FORMULATION AND PACKAGING*

B. coagulans LBSC is sold as a powder preparation of different spore counts, depending on the final intended application.

For the manufacturing of the dry spore preparation, the spray-dried unformulated concentrate (not less than 150 billion/g) is further formulated with approved food-grade formulating agents such as maltodextrin and adjusted to a declared spore count.

The *B. coagulans* LBSC preparation is tested by Quality Control for all quality related aspects and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for final preparations.

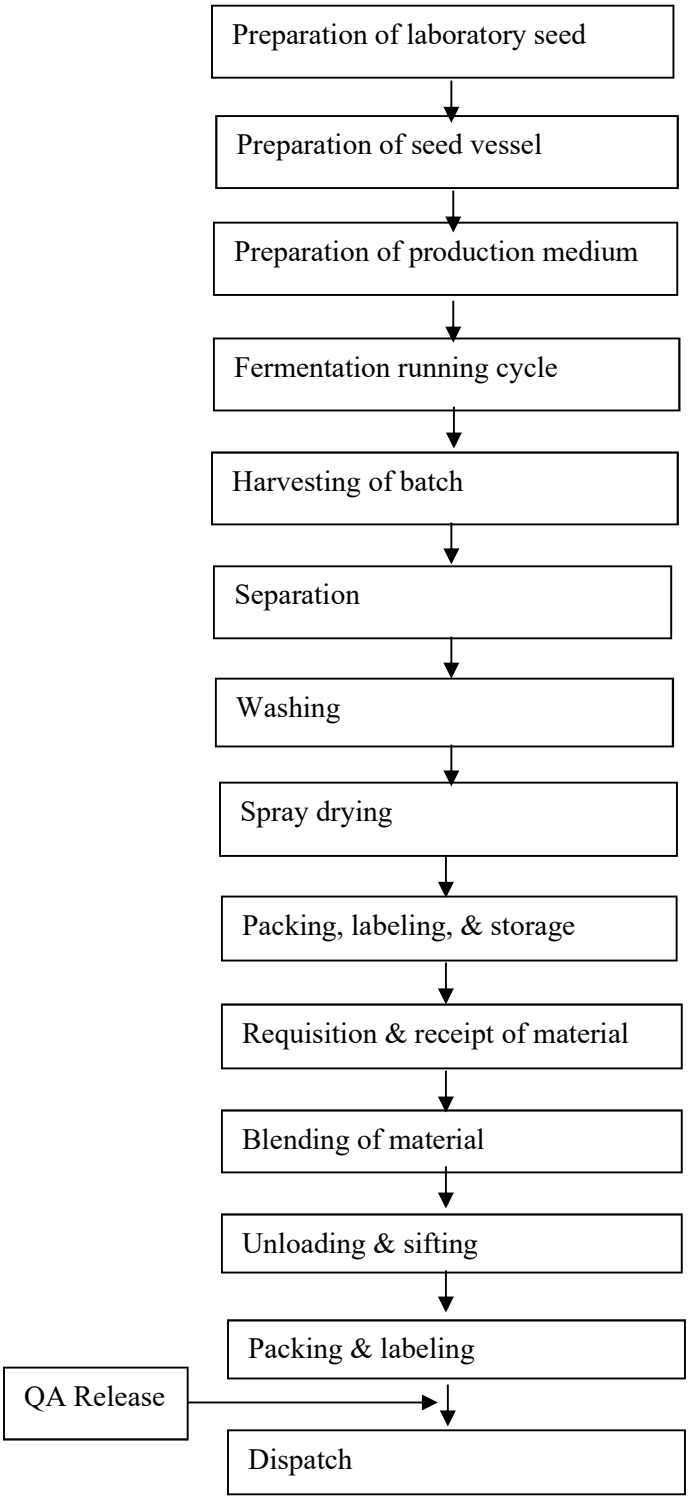

Figure 1. Manufacturing Process for *B. coagulans* LBSC

2.3 Product Specifications and Compositional Variability

2.3.1 *PRODUCT SPECIFICATIONS*

Specifications for *B. coagulans* LBSC preparation have been established by Advanced Enzyme Technologies Ltd. and are summarized in Table 4. All methods are current and have been validated for this purpose.

Table 4. Product Specifications for *B. coagulans* LBSC

Product specification	Advanced Enzyme Technologies Ltd.	
	Limits	Reference Method
Total viable count/ Assay (cfu/g)	Not less than 150 billion viable spore counts/g	Internal method
Appearance/ Description	Light brown to brown colored powder	Visual
Microscopy/ Identity	Gram positive rods with terminal spores	Internal method
Moisture/ Loss on Drying	Not more than 7.0%	AOAC 926.08
Sieve test	100% through 40 mesh	Internal method
Lactic acid producing capacity (ml)	Not less than 10 ml of 0.05 N NaOH should be consumed	Internal method
Arsenic	Not more than 2.0 ppm	USP
Cadmium	Not more than 1.0 ppm	USP
Lead	Not more than 3.0 ppm	USP
Mercury	Not more than 0.5 ppm	USP
Total bacterial counts	Not more than 0.2 million cfu/g	Harmonized method (IP,BP,EP and USP)
Total yeast & mold count	Not more than 100 cfu/g	Harmonized method (IP,BP,EP and USP)
Total coliform	Not more than 100 cfu/g	FDA Bacteriological Analytical Manual
E. coli	Absent in 10 g	Harmonized Pharmacopoeial method (EP, BP, USP, and IP)
Salmonella spp.	Absent in 10 g	Harmonized Pharmacopoeial method (BP, USP and IP)
P. aeruginosa	Absent in 1 g	Harmonized method (IP,BP,EP and USP)
Staphylococcus spp.	Absent in 1 g	Harmonized method (IP,BP,EP and USP)
Listeria monocytogenes	Absent in 25 g	Internal method

2.3.2 *COMPLIANCE WITH SPECIFICATIONS*

Three batches of *B. coagulans* LBSC were analyzed and the results compared with food-grade specifications. As shown in Table 5, all tested batches were in compliance, demonstrating that the production process is in control.

Table 5. Analysis of Compositional Variability of *B. coagulans* LBSC

Parameter	Specification	Batch		
		121857	121858	121859
B. coagulans viable spore count	Not less than 150 billion viable spore counts/g	172 billion viable spore count/g	165 billion viable spore count/g	170 billion viable spore count/g
Description	Light brown to brown colored powder	Light brown colored powder	Light brown colored powder	Light brown colored powder
Microscopy/ Identity	Gram positive rods with terminal spores	Complies	Complies	Complies
Sieve test	100% pass through 40 mesh.	Complies	Complies	Complies
Moisture/Loss on drying (%)	Not more than 7.0%	5.10	5.56	5.36
Lactic acid producing capacity	Not less than 10 ml of 0.05 N NaOH consumed	16.3 ml of 0.05N NaOH Consumed	16.2 ml of 0.05N NaOH consumed	16.0 ml of 0.05N NaOH consumed
Heavy Metal Analysis				
Arsenic	Not more than 2.0 ppm	Complies	Complies	Complies
Cadmium	Not more than 1.0 ppm	Complies	Complies	Complies
Lead	Not more than 3.0 ppm	Complies	Complies	Complies
Mercury	Not more than 0.5 ppm	Complies	Complies	Complies
Microbial Analysis				
Total Bacterial Count	Not more than 0.2 million cfu/g	13000 cfu/g	15000 cfu/g	11000 cfu/g
Total yeast & mold count	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
Total Coliform	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
E. coli	Absent in 10g	Complies	Complies	Complies
Salmonella	Absent in 10g	Complies	Complies	Complies
P. aeruginosa	Absent in 1g	Complies	Complies	Complies
Staphylococci	Absent in 1g	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25g	Complies	Complies	Complies

2.4 Shelf-Life Stability

Stability testing was performed on *B. coagulans* LBSC to assess its shelf life stability. In a real-time stability study, the samples were stored in an environmental chamber at 25°C ± 2°C and 60% ± 5% relative humidity for 36 months. In a accelerated stability study, samples were stored in an environ-mental chamber at accelerated storage conditions (40°C ± 2°C and 75% ± 5% relative humidity) for a period of six months.

B. coagulans LBSC was found stable for 36 months under real-time storage conditions and for 6 months under accelerated storage conditions. The real-time stability studies showed less than 15% loss of viable count; in the accelerated stability study, the activity drop of *B. coagulans* LBSC was less than 10%.

The shelf-life storage stability results obtained in the present studies corroborate the results presented in other GRAS notices for *B. coagulans* [GRN 399 (2011); GRN 526 (2014), GRN 597 (2015), GRN 601 (2015)]. These results suggests shelf life of *B. coagulans* LBSC is at least three years under real-time storage conditions when stored in simulated market packing [e.g. double polybag bag in HDPE drum (powder)].

Part 3: Intended Use and Dietary Exposure

B. coagulans LBSC is intended for addition at a level not exceeding $2x10^9$ cfu/serving to a wide variety of foods. Intended uses do not include infant formula or any foods under the jurisdiction of the U.S. Department of Agricuture. The food categories as defined in 21 CFR §170.3(n) to which *B. coagulans* LBSC is to be added are listed below:

(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours and mixes, requiring preparation before serving.

(2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix.

(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks, drinking water, sport drinks.

(4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.

(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.

(6) Chewing gum, including all forms.

(7) Coffee and tea, including regular, decaffeinated, and instant types.

(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.

(9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.

(10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.

(12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils.

(16) Fresh fruit juices, including only raw fruits, citrus, melons, and berries, and home prepared "ades" and punches made therefrom.

(20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties.

(21) Fruit and water ices, including all frozen fruit and water ices.

(22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.

(23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.

(25) Hard candy and cough drops, including all hard type candies.

(26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.

(28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.

(30) Milk, whole and skim, including only whole, low-fat, and skim fluid milks.

(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products.

The intended addition level of *B. coagulans* LBSC and the food caregories to which it will be added are identical to those stated for *B. coagulans* GBI-30, 6086 in GRN No. 000399, filed on August 23, 2011, to which FDA had no questions. This same addition level and intended uses were also specified in GRN No. 000526 for *B. coagulans* Unique IS2 (Unique Biotech, 2014), GRN No. 000597 for *B. coagulans* SNZ1969 (Sanzyme Ltd., 2014), and GRN No. 000691 for *B. coagulans* SANK 70258 (Mitsubishi, 2017). FDA had no questions regarding any of these GRAS determinations.

Because the intended use of *B. coagulans* LBSC matches these already existing use for other strains of *B. coagulans*, it merely represents an alternative strain with no increase in consumer exposure to the species. FDA accepted the EDI presented in GRN No. 000399, 36.4 x 10^9 cfu/day, which was also adopted in the succeeding GRAS notices for other strains of *B. coagulans*. Consequently, that same EDI is appropriate for *B. coagulans* strain LBSC.

Part 4: Self-Limiting Levels of Use

There are no self-limiting levels of use of *Bacillus coagulans* spores from *B. coagulans* LBSC in food applications.

Part 5: Experience Based on Common Use in Food before 1958

The statutory basis for our conclusion of GRAS status in the notice is scientific procedures rather than on common use in food prior to 1958.

Part 6: Narrative

6.1. History of Consumption of *Bacillus coagulans*

There is a long history of consumption of *Bacillus coagulans* in human food. Several commercial preparations containing *Bacillus coagulans*, as described in GRN 399, 526, 597, 601, 660, 691 are being used commercially as a food ingredient.

First mention of *B. coagulans* dates back to 1915 for coagulation of evaporated milk (Sarles and Hammer, 1932). Later in 1932, it was described as *Lactobacillus sporogenes*, responsible for curdling of milk with a cheesey flavor (Endres et al., 2009). Naruse and Naruse reported addition of *B. coagulans* along with *Bacillus natto* in Japanese soya-based fermented food preparation (USPTO No. 4,110,477, 1978).

B. coagulans has been isolated from traditional fermented foods such as bhaturu, chilra, Lugri, fermented milk, seera, cheese, and Jan chang from tribal areas of the western Himalayas (Sourabh et al. 2010). Salvetti et al. (2016) reported *B. coagulans* (AB553281.1) from fermented fish products. *B. coagulans* was found to be associated with a Nigerian fermented food, Ugba, prepared by fermentation of plant proteins from soya (An and Chuka, 2018; Grumezescu and Holban, 2017; Ogbadu et al., 1990). *Bacillus* cells in Ugba reached their highest population, $7.9x10^{11}$ cfu/g on the third day of fermentation (Isu and Njoku, 1997). Synergistic association of *B. coagulans* with other *Bacillus* spp. was reported by Adebayo-Tayo et al. (2013) in fermented foods like Iru, Aisa and Ugba. The worldwide usage of *B. coagulans* in fermented foods confims its safety for human consumption.

6.2 Regulatory History of *Bacillus coagulans*

Bacillus coagulans strains have long been known to be safely consumed by the general human population.

The European Food Safety Authority granted *B. coagulans* Qualified Presumption of Safety (QPS) status in 2008 (EFSA, 2007) and has renewed its status annually since then. Further, *B. coagulans* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC, as it is globally regarded as a safe microorganism.

The American Type Culture Collection (ATCC, 2020) has classified different strains of *B. coagulans* as Bio-safety Level 1, indicating that it is a well-characterized agent which does not cause disease in healthy humans.

The Food Safety and Standards Authority of India (FSSAI, 2016), includes *B. coagulans* in the list of permitted components in food (FSSAI, 2016).

Health Canada has issued a no objection letter to Ganeden for products fortified with *Bacillus coagulans* GBI-30 6086 (Food Business News, 2017). Health Canada has also permitted the use of *Bacillus coagulans* in the production of a glucose isomerase enzyme.

Food Standards Australia New Zealand (FSANZ) identified no safety concerns associated with *Bacillus coagulans*. (FSANZ, 2019).

The Japanese Ministry of Health and Welfare (JPCRF, 2020) has approved the use of *Bacillus coagulans* products for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998). *Bacillus coagulans* has a long history of approval in

Japan. *Bacillus coagulans* SANK 70258 spores preparation was launched by Sankyo Co., Ltd. in Japan for use in foods under the trade name LACRIS in 1966.

The U. S. Food and Drug Administration (FDA) issued a no questions letter (CFSAN, 2008) to a GRAS notice (GRN No. 000240) submitted by Purac (2008) for a fermented preparation of corn, cane, or beet sugar cultured with *Bacillus coagulans* LA-1 and other lactic acid producing bacteria for use in meat and poultry products at a level of 4.8%. *B. coagulans*, along with other microorganisms used in the preparation, were non-pathogenic, non-toxigenic organisms, commonly used in the food industry for the production of enzymes and cheese. The preparation containing *B. coagulans* LA-1 along with other microorganisms was used primarily as a food processing aid.

In another GRAS notice [GRN No. 000378 (2011)] submitted by Purac (2011), a similar preparation was presented. The cultured dairy sources, sugars, wheat, malt, and fruit and vegetable-based sources were fermented by *B. coagulans* LA-1 alongwith other lactic acid producing bacteria for use as antimicrobial agents in a variety of food categories at levels of 0.1 to 4.5%. All microorganisms including *B. coagulans* LA-1 have been mentioned as common food ingredients as well as in food processing aids. The FDA reviewed and responded that it had no questions (CFSAN, 2012a).

GRAS notification GRN No. 000399 was submitted to FDA by Ganeden Biotech, Inc., for *B. coagulans* GBI-30, 6086 (Ganeden, 2011). The submission considered *B. coagulans* GBI-30, 6086 addition to a wide variety of foods [as defined as 21 CFR 170.3(n)] at levels up to approximately $2x10^9$ cfu/serving to be GRAS. The food categories to which *B. coagulans* GBI-30, 6086 may be added included baked goods and baking mixes; alcoholic beverages; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy and cough drops; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups. Additional safety evidence in the form of history of human exposure and other scientific and evidence-based research that corroborates the safety of consumption of *B. coagulans* GBI-30, 608 was provided. The notification highlights the non-pathogenic and non-toxigenic nature of *B. coagulans* GBI-30, 6086. After its review, FDA responded to GRN No. 000399 with no questions (CFSAN, 2012b).

Another GRAS notification (GRN No. 000660) submitted by Ganeden Biotech, Inc., was for *B. coagulans* GBI-30, 6086 to be used in non-exempt term infant formula (Ganeden, 2016). The intended level of *B. coagulans* GBI-30, 6086 in non-exempt term infant formula was up to $2x10^8$ cfu/100 mL infant formula as ready for consumption. The notifier provided data on toxicological studies of *B. coagulans* GBI-30, 6086. FDA'S response to the notification was that the agency had no questions (CFSAN, 2017a).

Unique Biotech Ltd. submitted GRAS notification GRN No. 000526 (Unique Biotech, 2014). *B. coagulans* Unique IS2 spore preparation and its intended use was in various food categories with the exception of infant food formula, food for toddlers, at a maximum level of approximately 2×10^9 cfu/serving. Maximum daily intake of 36.4×10^9 cfu/day was claimed for the intended use. FDA did not have any questions in response to the notification (CFSAN, 2015a).

GRAS Notification (GRN 000597) for *B. coagulans* SNZ1969 spore preparation was submitted by Sanzyme Limited (2014); the intended use was identical to the that in GRN No. 000526. Conditions for intended use for *B. coagulans* spore preparation (LactoSpore®) remained identical in the GRAS Notification (GRN 000601) submitted by Sabinsa Corporation (Sabinsa Corporation, 2015). FDA had no questions in response to either of these notifications (CFSAN, 2016a; CFSAN, 2016b).

Mitsubishi Chemical Foods Corporation determined that the use of *B. coagulans* SANK 70258 as an ingredient in various food preparations at a maximum level of $2x10^9$ cfu/serving is GRAS (GRN No. 000691; Mitsubishi, 2017). The intended level of usage of *B. coagulans* spore preparation was calculated to yield an exposure of $36.4x10^9$ cfu/day. The green malt isolate, *B. coagulans* SANK 70258, is a Gram-positive, spore-forming, lactic-acid producing bacterium. *B. coagulans* SANK 70258 spore preparation was reported as nonpathogenic, nontoxigenic, nonmutagenic, and nongenotoxic and did not induce acute, subchronic, chronic, or reproductive toxicity in rats. *B. coagulans* SANK 70258 did not show treatment-related adverse events in human studies at levels up to $4x10^8$ cfu/day for eight weeks studied through a randomized, controlled clinical trial. FDA issued no questions regarding the GRAS notice (CFSAN, 2017b).

6.3 Safety of *Bacillus coagulans*—Oral Toxicity and Genotoxicity Studies

The safety of *Bacillus coagulans* LBSC and other strains have been evaluated in animal research, including acute, subacute, subchronic, and chronic studies of oral toxicity and genetic toxicity assays.

6.3.1. *STUDIES OF* BACILLUS COAGULANS *LBSC*

B. coagulans LBSC, the notified strain, has been investigated in a series of toxicity studies complying with OECD Guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP) as published by the OECD (1998).

Acute oral toxicity test (OECD Test No. 423, 2002): Using the step-wise method, 2 groups of n=3 female Wistar rats aged 8-10 weeks and weighing 195-221 g were dosed via gavage with 300 mg spore preparation ($5.13x10^4$ spores)/kg bw and observed for 14 days. No indications of toxicity were reported, and so 2 similar groups of n=3 female Wistar rats were gavaged with 2000 mg/kg bw of the spore preparation, providing $3.4x10^5$ spores/kg bw. Based on the results, the estimated LD_{50} for *B. coagulans* LBSC was greater than 2000 mg TOS/kg bw.

Repeated-dose 90-day oral toxicity test (OECD Test No. 408, 1998): Four groups of 10 male and 10 female Weistar rats, 7-8 weeks old and weighing 205-245 g (males, mean = 233.7 g) and 152-184 g (females, mean = 169.5 g) were assigned to receive daily oral gavage of doses of 0, 250, 500, and 1000 mg spore preparation/kg bw (providing 0, 0.43, 0.85, and $1.71x10^{11}$ spores/kg bw) for 90 days. Groups of 5 rats/sex receiving 0 or 1000 mg spore preparation/kg bw/day were assigned to 28-day recovery groups. Rats were examined daily for signs of toxicity, morbidity, and mortality. They were subjected to detailed clinical examinations at day 0 and weekly thereafter during the treatment and recovery period. Ophthalmic examinations were performed on the control and high-dose rats at beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes,

epididymides, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no mortality and no clinical abnormalities in rats treated at any dose. Ophthalmological examination revealed no abnormalities, nor did the neurotoxic assessment. There was no effect on feed intake or body weight gain, hematological or biochemical parameters, absolute or relative organ weights and no histopathology. The no observed adverse effect level (NOAEL) of B. coagulans spore preparation in the Wistar rat, following oral administration for 90 days, was the highest dose tested, 1000 mg/kg bw/day providing $1.71x10^{11}$ spores/kg bw/day. This study was published (Maity and Gupta 2019a).

Bacterial reverse mutation test—Ames assay (OECD Test No. 471, 1997): The test was conducted using *Salmonella typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA1535 in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 0, 50, 150, 500, 1500, and 5000 µg/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, *B. coagulans* LBSC spore preparation is non-mutagenic.

***In vitro* mammalian chromosomal aberration test in human lymphocytes (OECD Test No. 473, 2016):** Cutures of human peripheral blood lymphocytes were exposed to *B. coagulans* LBSC spore preparation at concentrations of 0, 200, 600, and 2000 µg/ml in the presence and absence of metabolic activation for 3 or 24 hours. No significant concentration related increase was reported in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that *B. coagulans* LBSC is non-clastogenic in the presence and absence of microsomal enzymes.

***In vivo* micronucleus test in mice (OECD Test No. 474, 2016):** Four groups of 5 male mice were gavaged with *B. coagulans* LBSC spore preparation at doses of 0, 500, 1000, and 2000 mg/kg bw on two consecutive days, after which bone marrow was aspirated and examined microscopically. A total of 4000 polychromatic erythrocytes per mouse were examined for the presence of micronucleated cells. No evidence of toxicity wa seen in treated mice or in their bone marrow with no increase in the incidence of micronucleated polychromatic erythrocytes. Based on the results obtained, it was concluded that *B. coagulans* LBSC is non-mutagenic under the conditions tested.

6.3.2. STUDIES OF OTHER STRAINS OF BACILLUS COAGULANS

B. coagulans SNZ 1969 was assessed for its safety through acute and sub-acute toxicity studies in Wistar rats (Metlakunta et al. 2020). In the acute oral toxicity study, rats were gavaged with 2000 mg *B. coagulans* SNZ 1969 spore preparation/kg bw, equivalent to 10^{12} cfu/kg bw. In the sub-acute (28-day) repeated-dose study of oral toxicity, groups of Wistar rats were adminstered 0, 50, 500, or 1000 mg/kg bw/day (maximum $5x10^{11}$ cfu/kg bw/day). There were no treatment related changes in any of the studied parameters, clinical signs, body weights, feed intake, urinalysis, hematological parameters, clinical biochemistries, gross pathology, and histopathology. The LD_{50} for *B. coagulans* SNZ 1969 was determined to be $>10^{12}$ cfu/kg bw and the NOAEL in the 28-day study was $5x10^{11}$ cfu/kg bw/day, the highest dose tested.

Acute and sub-acute oral toxicity tests of *B. coagulans* Unique IS-2 (MTCC-5260) were conducted in Sprague Dawley rats (Sudha et al. 2011a). The 36 rats (18/sex) in the acute study were gavaged with *B. coagulans* at 0, 1.6, or $3.2x10^{10}$ spores/kg bw and observed for 14 days.

The sub-acute (28-day) study included 48 Sprague Dawley rats, 24 rats/sex, which received gavage doses of 0, $6.5X10^8$, $3.25x10^9$, or $6.5 x10^9$ cfu/kg bw/day. There was no mortality and no treatment-related changes in clinical signs, body weight, feed intake, urinalysis, hematology, clinical chemistries, gross pathology, or histopathology exhibited by experimental rats at both time intervals. In these studies, the LD_{50} for *B. coagulans* Unique IS-2 (MTCC-5260) was $>3.2x10^{10}$ spores/kg bw and the sub-acute NOAEL was $6.5 x10^9$ cfu/kg bw/day, the highest dose tested.

An acute oral toxicity study in rats (5 rats/sex/dose) was performed by Endres et al. (2009) in Wistar Crl:(WI) BR rats. A single gavage dose of 0 or 5000 mg/kg bw of $1.04x10^{11}$ cfu/g of *B. coagulans* GBI-30, 6086 produced no treatment-related signs in any of the animals over the 14-day observation period. Neither weight-loss nor changes in body weight resulted with the treatment compared with the control group (administered with 1% methylcellulose). All of the organs examined in both the male and female dose groups were free from any gross pathological changes, and the LD_{50} for *B. coagulans* GBI-30, 6086 in Wistar rats was $>5.2x10^{11}$ cfu/kg bw.

Following the acute study, Endres et al. (2009) performed a 13-week subchronic study of oral toxicity with *B. coagulans* GBI-30, 6086. The strain was administered by gavage at doses of 0, $1.36x10^{10}$, $4.08x10^{10}$, or $1.36x10^{11}$ cfu/kg bw/day for 90 consecutive days to Wistar Crl:(WI)BR rats (10 rats/sex/group weighing 192-218 g [males] and 159-181 g [females]). There were no deaths, no treatment-related signs, and no toxicologically significant differences between the treated groups and the controls. The NOAEL for *B. coagulans* GBI-30, 6086 for male and female Wistar rats in this study was the highest dose tested, $1.36x10^{11}$ cfu/kg bw/day.

Endres et al. (2009) also performed bacterial reverse mutation assays, mouse micronucleus assays, and *in vitro* chromosomal aberration assays on the strain and determined that *B. coagulans* GBI-30, 6086 "does not demonstrate mutagenic, clastogenic, or genotoxic effects.

A chronic one-year oral toxicity study was was reported by Endres et al. (2011) for *B. coagulans* GBI-30, 6086 in male and female HsdBrlHan rats of Wistar origin, 7-9 weeks of age and weighing 175-217 g (males) and 136-189 g (females). Groups of 20rats/sex were fed chow containing levels of the test article providing target doses of 0, 600, 1200 and 2000 mg/kg bw/day. All groups were fed diets containing the stipulated concentration of test product for 52 to 53 weeks on a daily dosing schedule of a 7-day per week basis. No mortality occurred in any of the male groups (control, 600, 1200 and 2000 mg/kg bw/day) or the female group receiving 600 mg/kg bw/day of the test article. One female treated with 1200 mg/kg bw/day was found dead on day 137. The cause of death was attributed to an individual disorder. No effect of the test article was noted on body weight development. No toxicologically relevant differences in hematological parameters in any treated groups were found at the end of the 12 months. Histological examination did not reveal any test article-related or toxic lesions in the investigated organs. The authors concluded that *B. coagulans* GBI-30, 6086 "caused no signs of toxicity in male or female HsdBrlHan: Wistar rats after one year of diet-mixed administration. The NOEL was 1948 mg/kg bw/day for the males and 2525 mg/kg bw/day for the females – the highest dose tested."

6.4 Safety of *Bacillus coagulans*—Human Studies

Several researchers carried out studies with different *Bacillus coagulans* strains on human subjects and evaluated the safety aspects. These studies are summarized in Table 6.

Table 6. Human Studies of *Bacillus coagulans*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Adults					
Ara et al. (2002)	Open-label study	20 apparently healthy adults (16M, 4F) aged 20-40 years	*Bacillus coagulans* SANK 70258 at a dose of 10^8 cfu/day	2 weeks	All effects on defecation frequency; fecal form, microbiota, pH, odor, enzyme concentration, decomposition products, and color; feeling after defecation; and dermal effects were regarded as beneficial with no adverse effects.
Kalman et al. (2009)	Prospective, randomized, double-blind, placebo-controlled, multicenter trial of effects on GI symptoms	61 otherwise healthy adults aged 36.5±12.6 years with post-prandial intestinal gas-related symptoms	*Bacillus coagulans* GBI-30, 6086; 1 capsule providing $2x10^9$ cfu/day	4 weeks	Based on questionnaires and biochemical testing, but no discussion of the latter. No adverse events; lower scores than placebo on self-assessed abdominal pain, distention, flatus, bloating, and gas.
Hun (2009) [1st study]	Prospective, randomized, double-blind, placebo-controlled pilot study of treatment of patients with irritable bowel syndrome (IBS)	52 patients with diarrhea-predominant IBS	*B. coagulans* GBI-30, 6086	8 weeks	Self-assessments of the severity of IBS symptoms (abdominal pain and bloating) were recorded every day. No treatment-related AEs or SAEs were reported.
Hun (2009) [2nd study]	Prospective, randomized, double-blind, placebo-controlled study of treatment of patients with D-IBS	44 D-IBS patients	*B. coagulans* GBI-30, 6086	8 weeks	Self-assessments of the severity of IBS symptoms (abdominal pain and bloating) were recorded every day for 8 weeks. No treatment-related AEs or SAEs were reported. The author concluded that, "*B. coagulans* GBI-30, 6086 . . . may be a safe and effective option for the relief of abdominal pain and bloating for pa ients ith IB

Table 6. Human Studies of *Bacillus coagulans*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Dolin (2009)	Prospective, randomized, double-blind, placebo-controlled pilot study of treatment of patients with D-IBS	61 patients with D-IBS	*B. coagulans* GBI-30, 6086	8 weeks	Self-assessments of stool counts, severity of IBS symptoms, and use of medication. were recorded every day. The authors concluded that (the proprietary B. coagulans GBI-30, 6086 is safe and effective for reducing daily bowel movements in patients with IBS- .
Mandel et al. (2010)	Prospective, randomized, double-blind, placebo-controlled trial of treatment of rheumatoid arthritis with microorganisms	45 patients of both sexes aged 36-82 years (mean age = 62.5 years) with rheumatoid arthritis	*B. coagulans* GBI-30, 6086, dose = $2x10^9$ cfu/ day	60 days	There were no treatment-related AEs and no SAEs reported (4 reported AEs were shingles, poison ivy, a cold, and leg edema ive treatmen ith *B. coagulans* GBI-30, 6086 appeared to be . . . well tolerated by the study popul i
Sudha et al. (2011b)	Prospective, randomized, double-blind, placebo-controlled trial to test effect of *B. coagulans* strain Unique IS-2 on patients with hypercholesterolemia	30 hyperlipidemic subjects divided into 3 groups (n = 10)	*B. coagulans* strain Unique IS-2, dose = 0, 10^{10}, or 10^{11} cfu/day	60 days	No adverse events or serious adverse events were reported.
Sudha and Bhonagiri (2012)	Open-label study of the efficacy and safety of *B. coagulans* strain Unique IS-2 for treatment of acute diarrhea	28 patients aged 18-55 years with acute diarrhea	*B. coagulans* strain Unique IS-2, dose = $4x10^9$ cfu/day	10 days	Safety was evaluated by assessment of incidence and type of AEs, physical examination, and clinical laboratory test values such as complete blood count, serum glutamic pyruvic transaminase, serum creatinine, stool routine, and microscopy on days 1,3, 6, and 10. No significant change in safety parameters was observed.

Table 6. Human Studies of *Bacillus coagulans*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Majeed et al. (2016)	Prospective, randomized, double-blind, placebo-controlled trial to test tolerance and safety of *B. coagulans* MTCC 5856 in treatment of diarrhea-predominant IBS	36 newly diagnosed D-IBS patients; 17M & 19F; mean age = 35.8±10.9 years	*B. coagulans* MTCC 5856, $2x10^9$ cfu/day	90 days	All safety parameters (blood hematology and clinical chemistry parameters), anthropometric measures (weight, BMI, blood pressure and heart rate) and vital sign measures remained within normal clinical range during the 90-day study. No additional AEs were observed in the test group when compared with the placebo gro . luded that the *B. coagulans* MTCC 5856 at a dose of 2×10^9 cfu/day along with standard care of treatment was found to be safe and effective in diarrhea predominant IBS patients for 90 days of supplement i .
Majeed et al. (2018)	Prospective, randomized, double-blind, placebo-controlled multicenter pilot trial to assess safety and efficacy of *B. coagulans* MTCC 5856 for major depressive disorder (MDD) in IBS patients	40 IBS patients with MDD, 6M & 34F, mean age = 42.1±10.1 years	*B. coagulans* MTCC 5856, dose = $2x10^9$ cfu/day	90 days	Vital signs (blood pressure, respiratory rate, pulse rate, abnormal lab/diagnostic parameters) were considered for safety evaluations. No clinically significant changes were recorded in physical examinations. No clinically significant abnormal lab values (biochemistry and hematology) were identified, and no statistically significant changes in the vitals were observed from the baseline to final visit. No serious AEs or significant AEs were noticed in this study. There was only one AE reported, fever and weakness, in the placebo group.

Table 6. Human Studies of *Bacillus coagulans*

Jager et al. (2018)	Prospective, randomized, single-blind crossover trial of the effect of *B. coagulans* GBI-30, 6086 on absorption of selected amino acids	29 apparently healthy athletes who consumed a diet high in whey protein	*B. coagulans* GBI-30, 6086, dose = 10^9 cfu/ day	2 weeks	Reporting of findings was generally inadequate, and no mention was made of any adverse effects or AEs.
Madempudi et al. (2019a)	Prospective, randomized, double-blind, placebo-controlled trial of use of *B. coagulans* Unique IS2 in treatment of IBS patients	108 IBS patients (78M, 30F) aged 20-60 years (median = 45 years)	*B. coagulans* Unique IS2, $2x10^9$ cfu/day	8 weeks	Hematology of both the arms remained normal. No significant changes were reported in pro-inflammatory cytokines IL-6, IL-12, TNF-α, INF- γ or anti- 10. “ *coagulans* was well tolerated with no SAE
Madempudi et al. (2019b)	Prospective, randomized, double-blind, placebo-controlled trial of the efficacy of *B. coagulans* in treating functional constipation	100 subjects with functional constipation	*B. coagulans* Unique IS2, $2x10^9$ cfu/day	4 weeks	No adverse effects such as painful evacuation or abdominal pain were reported.

Table 6. Human Studies of *Bacillus coagulans*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Gupta et al. (2020; unpublished)	Prospective, randomized, double-blind, placebo-controlled trial of interven-tion for IBS	IBS patients (Rome IV criteria) aged 18-65 years	*B. coagulans* LBSC (DSM17654), dose = $2x10^9$ cfu/day	80 days	No intervention-associated AEs and no SAEs were reported. Vital, biochemical and hematological parameters were within normal range. Upper GI endoscopy revealed no clinical changes of GI mucosa on *B. coagulans* LBSC supplementation.
Infants					

Table 6. Human Studies of *Bacillus coagulans*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Labalestra et al. (2008)	Prospective, randomized, double-blind, placebo-controlled crossover trial of *B. coagulans* in treatment of gastroesophageal reflux (GER)	19 children (8M, 11F) under 1 year of age (mean age = 5.5 months) with symptomatic GER	*B. coagulans* + symeth-icone; strain and dosage not reported	7 days	No reported AEs or SAEs.
Dutta et al. (2011)	Prospective, randomized, double-blind, placebo-controlled trial to assess the clinical efficacy of *Bacillus coagulans* against dehydrating diarrhea in children	148 boys aged 6-24 months with diarrhea	*B. coagulans* tablets with $2.4x10^8$ cfu/day	5 days	Children were examined daily. The study product was well received and tolerated by all children. The authors stated that, "No adverse event or complica ion was observed during hospital stay and during follow up period of 15 days after ischar
Maity and Gupta (2019b)	Prospective, randomized, double-blind, placebo-controlled trial of *Bacillus coagulans* treatment for acute diarrhea in children	60 children with acute diarrhea suffering stomach discomfort	*B. coagulans* LBSC at $6x10^9$ cfu/day	To resolution of diarrhea	Safety assessment involved physical examination and vitals, hematological analysis, and assessment of reported AEs or SAEs. Data showed that the *B. coagulans* LBSC was safe and well-tolerated by participants at the dose provided. No treatment-associated AEs or SAEs were reported during the study.

6.5 Decision Tree

The safety of *B. coagulans* LBSC has also been established using the decision tree for determining safety of microbial culture to be consumed by Humans or Animals (Pariza et al. 2015)

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? **YES**

2. Has the strain genome been sequenced? **YES**

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? **YES**

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? **YES**

5. Does the strain produce antimicrobial substances? **NO**

6. Has the strain been genetically modified using rDNA techniques? **NO**

7. Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? **NO.** (The strain was isolated from soil.)

8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? **NO**

Conclusion: The strain is "deemed to be safe for use in the manufacture of food, probiotics, . . . for human consumption" (Pariza et al., 2015).

6.6 Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of *B. coagulans* LBSC spore preparation is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of *B. coagulans* LBSC is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to *B. coagulans* LBSC under its intended conditions of use is not harmful. In the second step, the intended use of *B. coagulans* LBSC is determined to be GRAS by demonstrating that the safety of this spore preparation under its intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or microorganism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1. Data and information relied upon to establish the scientific element of safety must be generally available; and
2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the intended use of *B. coagulans* LBSC spore preparation is safe and is GRAS.

6.6.1 *EVIDENCE OF SAFETY*

Genomic analysis of *B. coagulans* LBSC established that it harbors no antibiotic resistance genes flanked by mobile elements, no confirmed virulence genes and none flanked by mobile elements, and no genes encoding toxin production. Phenotypic analysis shows an absence of antibiotic resistance above ECOFF levels and no production of biogenic amines. No evidence of pathogenicity has been reported, and the species is generally regarded as non-pathogenic as well as non-toxigenic. No indications of toxicity were found in acute and repeated-dose studies of oral toxicity or in genotoxicity assays in strain LBSC or other strains of *B. coagulans*, and no adverse effects were reported when the spores are administered to humans. All of these findings support the conclusion that the intended use of *B. coagulans* LBSC spore preparation is safe.

6.6.2 *CONCLUSION OF THE EXPERT PANEL*

The intended use of *B. coagulans* LBSC spore preparation has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by genomic analysis of the strain, a record of safe ingestion of numerous strains of *B. coagulans*, toxicity studies of *B. coagulans* LBSC and other strains, and research in humans, concluding that the expected exposure to *B. coagulans* LBSC spore preparation is without significant risk of harm. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the intended use of *B. coagulans* LBSC spore preparation has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by Advanced Enzyme Technologies, as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They independently critically reviewed and evaluated the publicly available information and the potential human exposure to *B. coagulans* LBSC spore preparation anticipated to result from its intended use, and individually and collectively determined that no evidence exists in the available information on *B. coagulans* LBSC that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *B. coagulans* LBSC spore preparation.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion regarding the safety of *B. coagulans* LBSC under its intended conditions of use. Therefore, the intended use of *B. coagulans* LBSC spore preparation is GRAS by scientific procedures.

6.7. Affirmative Statement Concerning Data and Information

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with Advanced Enzyme Technologies' conclusion of GRAS status under the conditions of intended use.

(b) (6)

Part 7: References

Adebayo-Tayo B, Elelu T, Akinola G, Oyinloye I. 2013. Screening and production of mannanase by *Bacillus* strains isolated from fermented food condiments. *Innovat Roman Food Biotechnol* 13:53.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Molec Biol* 215:403-410.

American Type Culture Collection (ATCC). 2020. *Biosafety levels*. https://www.atcc.org/search#q=bacillus%20coagulans&sort=relevancy.

Ara K, Meguro S, Hase T, Tokimitsu I, Otsuji K, Kawai S, Ito S, Iino H. 2002. Effect of spore-bearing lactic acid-forming bacteria (*Bacillus coagulans* SANK 70258) administration on the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics in humans and rats. *Microb Ecol Health Dis* 14:4-13.

Bankevich A, Sergey N, Dmitry A, Alexey AG, Mikhail D, Alexander SK, Valery ML, Sergey IN, Son P, Andrey DP, Alexey VP, Alexander VS, Nikolay V, Glenn T, Max AA, Pavel AP. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Computa Biol* 19:455-477.

CFSAN. 2008. Agency Response Letter GRAS Notice No. GRN 000240, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=240

CFSAN. 2012a. Agency Response Letter GRAS Notice No. GRN 000378, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=378

CFSAN. 2012b. Agency Response Letter GRAS Notice No. GRN 000399, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=399

CFSAN. 2015a. Agency Response Letter GRAS Notice No. GRN 000526, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=526

CFSAN. 2016a. Agency Response Letter GRAS Notice No. GRN 000597, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=597

CFSAN. 2016b. Agency Response Letter GRAS Notice No. GRN 000601, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=601

CFSAN. 2017a. Agency Response Letter GRAS Notice No. GRN 000660, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=660

CFSAN. 2017b. Agency Response Letter GRAS Notice No. GRN 000691, FDA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=691

Clinical and Laboratory Standards Institute (CLSI). 2012. M100-S25. *Performance standards for antimickrobial susceptibility*, 25th informational supplement.

Dolin BJ. 2009. Effects of a proprietary *Bacillus coagulans* preparation on symptoms of diarrhea-predominant irritable bowel syndrome. *Exp Clin Pharmacol* 31:655-659.

Dutta P, Mitra U, Dutta S, Rajendran K, Saha TK, Chatterjee MK. 2011. Randomised controlled clinical trial of *Lactobacillus sporogenes (Bacillus coagulans*), used as probiotic in clinical practice, on acute watery diarrhoea in children. *Trop Med Int Health* 16:555–561.

EFSA. 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *EFSA Journal* 587:1-16 http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2007.587/epdf

EFSA. 2012. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal* 10:2740.

EFSA. 2014. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. *EFSA Journal* 12:3665.

Endres JR, Clewell A, Jade KA, Farber T, Hauswirth J, Schauss AG. 2009. Safety assessment of a proprietary preparation of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem Toxicol* 47:1231-1238.

Endres JR, Qureshi I, Farber T, Hauswitth J, Hirka G, Pasics I, Schauss AG. 2011. One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem Toxicol* 49:1174-1182.

Food Business News. 2017. *Ganeden receives Health Canada letter of approval*. August 16.

Food Safety and Standards Authority of India. 2016. *Health supplement, nutraceuticals, FSDU, FSMP and Novel Food Regulations*. https://fssai.gov.in/dam/jcr:0397428f-549b-448b-9466-8effcd9769f7/Direction_Operationalisation_HS_SMP_NF_Nutra_24_11_2016.pdf

FSANZ. 2019. *Record of views formed in response to novel food inquiries*. http://www.foodstandars.gov.au/industry/novel/novelrecs/Documents

Ganeden Biotech 2011. GRN 399. *Notice to US Food and Drug Administration that Bacillus coagulans GBl-30, 6086, a Novel Probiotic, is Generally Recognized as Safe for use in Foods*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=399

Ganeden Biotech. 2016. GRN 660. *GRAS notification for. Bacillus coagulans GBI-30, 6086 for use in Non-exempt Term Infant Formula*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=660

Grumezescu AM, Holban AM. 2017. *Soft chemistry and food fermentation*. Academic Press.

Hammer BW. 1915. Bacteriological studies on the coagulation of evaporated milk. *Iowa Agric Exp Stn Res Bull* 19:119-131.

Hun L. 2009. *Bacillus coagulans* significantly improved abdominal pain and bloating in patients with IBS. *Postgrad Med* 121:119-124.

Isu NR, Njoku HO. 1997. An evalution of the microflora associated with fermented African oil bean (*Pentaclethra macrophylla* Bentham) seeds durin ugba production. *Plant Foods Hum Nutr* 51:145–157.

Jäger R, Purpura M, Farmer S, Cash HA, Keller D. 2018. Probiotic *Bacillus coagulans* GBI-30, 6086 improves protein absorption and utilization. *Probiot Antimicrob Prot* 10:1–5.

Japanese Food Chemical Research Foundation (JFCRF). 2020. List of substances which are generally provided for eating or drinking as foods and which are used as food additives. https://

www.ffcr.or.jp/en/tenka/list-of-substances/list-of-substances-which-are-generally-provided-for-eating-or-drinking-as-foods-and-which-are-used-a.html

Kalman D, Schwartz H, Alvarez P, Feldman S, Pezzullo JC, Krieger DR. 2009. A prospective, randomized, double-blind, placebo-controlled parallel-group dual site trial to evaluate the effects of a *Bacillus coagulans*-based product on functional intestinal gas symptoms. *BMC Gastroenterol* 9:1-7.

Kolmogorov M, Raney B, Paten B, Pham S. 2014. Ragout. A reference-assisted assembly tool for bacterial genomes. *Bioinformatics* 30:i302–i309.

Labalestra V, Pannone V, Iacono O, Federici T, Maiella G, Oliva S, Tricarico A, Paganelli M, Cucchiara S. 2008. Effect of a combination of symethicone and *Bacillus coagulans* (Colinox) on the gastric emptying time in infants with symptomatic gastroesophageal reflux (GER): A randomized, double-blind, placebo-controlled, cross-over study. *Digest Liver Dis* 40:A72.

Leplae R, Mendez G, Toussaint A 2010. ACLAME: A CLAssification of Mobile genetic Elements, update 2010. *Nucleic Acids Res* 38:D57–D61.

Logan N, Vos P. 2015. *Bacillus; Firmicutes / "Bacilli" / Bacillales / Bacillaceae / Bacillus*; Cohn 1872, 174AL; Bergey's Manual of Systematics of Archaea and Bacteria.

Madempudi RS, Ahire JJ, Neelamraju J, Tripathi A, Nanal S. 2019a. Randomized clinical trial: the effect of probiotic *Bacillus coagulans* Unique IS2 vs. placebo on the symptoms management of irritable bowel syndrome in adults. *Sci Rep* 9:12210.

Madempudi RS, Neelamraju J, Ahire JJ, Gupta SK, Shukla VK. 2019b. *Bacillus coagulans* Unique IS2 in constipation: a double-blind, placebo-controlled study. *Probiot Antimicrob Proteins* Mar 25.

Maity C, Gupta AK. 2019a. Safety studies of probiotic *B. coagulans* LBSC in rat. *Proc Int Sci Conf Probiot, Prebiot, Gut Microbiota Health*:185.

Maity C, Gupta AK. 2019b. A prospective, interventional, randomized, double-blind, placebo-controlled clinical study to evaluate the efficacy and safety of *Bacillus coagulans* LBSC in the treatment of acute diarrhea with abdominal discomfort. *Eur J Clin Pharmacol* 75:21-31.

Majeed M, Nagabhushanam K, Arumugam S, Majeed S, Ali F. 2018. *Bacillus coagulans* MTCC 5856 for the management of major depression with irritable bowel syndrome: A randomised, double-blind, placebo controlled, multi-centre, pilot clinical study. *Food Nutr Res* 62:1218–1232.

Majeed M, Nagabhushanam K, Natarajan S, Sivakumar A, Ali F, Pande A, Karri SK. 2016. *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant irritable bowel syndrome: A double blind randomized placebo controlled pilot clinical study. *Nutr J* 15:21–31.

Majeed M, Prakash L. 1998. *Lacrospore®: The effective probiotic*. Piscataway, NJ: Nutri Science Publishers, Inc.

Mandel DR, Eichas K, Holmes J. 2010. *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Comple Altern Med* 12:1.

Meier-Kolthoff JP, Klenk HP, Göker M. 2014. Taxonomic use of DNA G+C content and DNA–DNA hybridization in the genomic age. *Int J Syst Evol Microbiol* 64:352–356.

Metlakunta AS, Soman RJ 2020. Safety evaluation of *Bacillus coagulans* SNZ 1969 in Wistar rats. *Regul Toxicol Pharmacol* 110:104538.

Mitsubishi. 2017. GRN 691. *GRAS Conclusion for the Use of Bacillus coagulans SANK 70258 Spores Preparation (LACRIS-S) in Select Foods*. Mitsubishi. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=691

OECD. 1998. *Principles on Good Lab Practice Series on Principles of Good Laboratory Practice and Compliance Monitoring*; 1-41. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/MC/CHEM(98)17&docLanguage=En

OECD. 1998. Test No. 408: *Repeated dose 90-day oral toxicity study in rodents* OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-10 | http://www.oecd ilibrary.org/docserver/download/9740801e.pdf?expires=1406712563&id=id&accname=guest&checksum=89F81777E38FA1ED3B9FFAC5CA5A2E49

OECD. 1997. Test No. 471: *Bacterial Reverse Mutation Test* OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-11 http://www.oecd-ilibrary.org/docserver/download/9747101e.pdf?expires=1406711627&id=id&accname=guest&checksum=A1D7097B5DDBAD66775DC1968812499D

OECD. 2016. Test No. 473: *In Vitro Mammalian Chromosome Aberration Test* OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-22 | https://www.oecd ilibrary.org/docserver/9789264264649en.pdf?expires=1548330475&id=id&accname=guest&checksum=092729F6D7AE0112C8104E15C309A3CD

OECD. 2016. Test No. 474: *In Vivo Mammalian Erythrocyte Micronucleus Test* OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-21. https://www.oecdilibrary.org/docserver/9789264264762en.pdf?expires=1548330284&id=id&accname=guest&checksum=94F500B893A095D94BAD725BAF52C4A7

Ogbadu LJ, Okagbue RN, Ahmad AA. 1990. Glutamic acid production by *Bacillus* isolates from Nigerian fermented vegetable proteins. *World J Microbiol Biotechnol* 6:377–382.

Pariza MW, Gillies KO, Kraak-Ripple SF, Leyer G, Smith AB. 2015. Determining the safety of microbial cultures for consumption by humans and animals. *Regul Toxicol Pharmacol* 73:164-171.

Purac. 2008. GRN 240. *GRAS Notification for VERDAD for use as a flavoring agent and antimicrobial agent in meat and poultry products*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=240

Purac. 2011. GRN 378. *GRAS Notification for Food Ferment Solutions*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=378

Reed R, Murray EGD, Smith NR. 1957. *Bergey's manual of determinative bacteriology*. Seventh Ed. The Williams and Wilkins Company, Baltimore.

Sabinsa Corp. 2015. GRN 601. *GRAS Notification for Bacillus coagulans SBC37-01 spores preparation*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=601

Salvetti E, Orrù L, Capozzi V, Martina A, Lamontanara A, Keller D, Spano G. 2016. Integrate genome-based assessment of safety for probiotic strains: *Bacillus coagulans* GBI-30, 6086 as a case study. *Appl Microbiol Biotechnol* 100:4595-4605.

Sanzyme Ltd. 2014. GRN 597. *GRAS Notification for Bacillus coagulans SNZ1969 spore preparation*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=597

Sarles WB, Hammer BW. 1932. Observations on *Bacillus coagulans*. *J Bacteriol* 23:301-314.

Saroj DB, Gupta AK. 2020. Genome based safety assessment for *Bacillus coagulans* strain LBSC (DSM 17654) for probiotic application. *Int J Food Microbiol* 318:108523

Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34:D32-36.

Simpson JT, Durbin R. 2012. Efficient *de novo* assembly of large genomes using compressed data structures. *Genome Res* 22:549-556.

Sourabh A, Kanwar S, Sharma P. 2010. Diversity of bacterial probiotics in traditional fermented foods of Western Himalayas. *J Probiot Prebiot* 5:193-202.

Sudha R, Sunita M, Sekhar B. 2011a. Safety studies of *Bacillus coagulans* Unique IS-2 in rats: morphological, biochemical and clinical evaluations. *Int J Probiot Prebiot* 6:43-48.

Sudha RM, Radkar N, Maurya A. 2011b. Effect of supplementation of probiotic *Bacillus coagulans* Unique IS-2 (ATCC PAT-11748) on hypercholesterolemic subjects: a clinical study. *Int J Probiot Prebiot* 6:89-93.

Sudha RM, Bhonagiri S. 2012. Efficacy of *Bacillus coagulans* strain Unique IS-2 in the treatment of patients with acute diarrhea. *Int J Probiot Prebiot* 7:33-37.

Sudha RM, Chauhan P, Dixit K, Babu S, Jamil, K. 2010. Molecular typing and probiotic attributes of a new strain of *Bacillus coagulans*-Unique IS-2: a potential biotherapeutic agent. *Genetic Engineer Biotechnol J* GEBJ-7:1-20.

Unique Biotech. 2014. GRN 526. *GRAS Notification for Bacillus coagulans Unique 1S2.* https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=526

USP. 2008. *Combatting antimicrobial resistance*. USP Global Public Policy Position. www.usp.org.

USPTO No. 4,110,477. 1978. *Method for producing natto containing lactic acid bacteria*. Naruse and Naruse.

Zhang D, Zhang H, Li T, Chen K, Qiu JL, Gao C. 2017. Perfectly matched 20-nucleotide guide RNA sequences enable robust genome editing using high-fidelity SpCas9 nucleases. *Genome Biol* 18:191.

From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Cc: "Ankit Rathi"
Subject: RE: GRN 000949 - Questions for Notifier
Date: Tuesday, December 1, 2020 8:17:40 AM
Attachments: image001.png
Hice Stephanie 20201201.pdf
Attachment I.pdf
Attachment II.pdf
Attachment III.pdf
Attachment IV Analyses of Non-Consecutive Batches.pdf

Dear Dr. Hice—

I hope you had a pleasant Thanksgiving. Attached please find our responses to the FDA questions. I've attached a letter providing the responses along with three attachments with excerpts from cited documents and one attachment containing the results of analyses of three non-consecutive batches of product. In this last attachment, the table shows the reduction of the specifications for heavy metals to 0.5 mg/kg.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Friday, November 20, 2020 3:47 PM
To: jh@jheimbach.com
Subject: GRN 000949 - Questions for Notifier

Dear Dr. Heimbach,

During our review of GRAS Notice No. 000949, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your response.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

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JHeimbach LLC

December 1, 2020

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Dear Dr. Hice:

On Friday, November 20, you sent me an email listing 14 questions raised by FDA reviewers regarding GRN 949 for *Bacillus coagulans* strain DSM 17654 and requested a reply within ten business days.

My clients and I offer the following in response to your questions.

1. For the administrative record, please describe whether *Bacillus coagulans* strain DSM 17654 produces antibiotics.

As was stated in the GRAS notice (Section 2.1.5 Antimicrobial Activity), *B. coagulans* strain DSM 17654 was evaluated for its antimicrobial activity following 2012 CLSI guidelines and the U.S. Pharmacopoeia. The strain showed an absence of antimicrobial activity against the select test microorganisms. We therefore conclude that *B. coagulans* strain DSM 17654 does not produce antibiotics.

2. Please state whether *B. coagulans* strain DSM 17654 is genetically engineered.

B. coagulans strain DSM 17654 is not genetically engineered.

3. On page 7, the notifier states “Through the above analysis, 144 putative antibiotic resistance genes were identified”, however, the notifier only lists a total of 143 putative antibiotic resistance genes. Please clarify this discrepancy.

Mention of 144 putative antibiotic resistance genes was a typographical error for 143. We apologize for the error.

4. On page 8, the notifier states "To confirm the above analysis, *B. coagulans* strain LBSC was tested as per CLSI guidelines for its sensitivity/resistance against nine antibiotics, ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol"; however, on page 12, the notifier states "*B. coagulans* LBSC was found to be sensitive to all eight tested antibiotics". For the administrative record, please clarify this discrepancy.

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The discussion of antibiotic testing in Section 2.1.2.1.e (page 8) was in error. Although the CLSI guidelines do include testing for nine antibiotics, as explained in Section 2.1.3 on page 12, the guidelines for testing of *Bacillus* species do not call for inclusion of ampicillin. For this reason, *B. coagulans* strain DSM 17654 was tested only against the remaining eight antibiotics.

5. On page 8, the notifier states, “A total of 76 virulence factor proteins were found to have significant homology with the assembled genome”, however, the notifier only lists a total of 75 virulence factor proteins. Please clarify this discrepancy.

The discrepancy is the result of the accidental omission of a single uncharacterized conserved protein, which brings the count up to the stated total of 76 virulence factor proteins.

6. On page 12, the notifier states, "Both *B. coagulans* LBSC and the control were sensitive to the aforementioned antibiotics. It was also observed that both *B. coagulans* LBSC and the control were resistant to aztreonam, cefonicid, and ceftazidime at the tested antibiotic concentrations". Please describe whether this poses a potential safety concern.

We do not believe that resistance to these three antibiotics poses a risk to the safety of the intended use of *B. coagulans* strain DSM 17654. Antibiotic resistance is common and intrinsic in *Bacillus* species. It is reported in other *B. coagulans* strains such as *B. coagulans* strain SNZ1969 (GRN 597), which showed resistance to cefuroxine, metronidazole, cefaclor, cefoxitin, colistin, novobiocin, and metronidazole. *B. coagulans* strain GBI-30 6086 (GRN 660), is reported to be resistant to kanamycin and streptomycin.

Bacillus spp. are known to show resistance to beta-lactam antibiotics due to the presence of a beta-lactamase gene intrinsic to the genome. The antibiotics aztreonam, cefonicid, and ceftazidime, to which *B. coagulans* strain DSM 17654 is resistant, are beta-lactam antibiotics. The resistance of *B. coagulans* strain DSM 17654 to these antibiotics is likely due to the presence of intrinsic beta-lactamase gene (protein id CIW84_15135) present in the genome of the strain. This resistance does not pose any safety concern as this gene is intrinsic.

B. coagulans strains have been reported to lack plasmids and are consequently unable to transfer antibiotic resistance genes, a fact noted in GRN 691. Genomic analysis of *B. coagulans* strain DSM 17654 did not reveal presence of plasmids; therefore, the strain is unlikely to transfer any antibiotic resistance and does not pose a safety concern in this respect.

7. Please specify how the purity of the initial inoculum is ensured.

Initial inoculum purity of *B. coagulans* strain DSM 17654 is ensured by validated growth pattern, direct microscopic evaluation, and characteristic viable growth on the agar medium.

8. In Table 4 (page 18), the notifier lists the reference method for total bacterial counts, total yeast and mold count, *Escherichia coli*, *Salmonella* serovars, *Pseudomonas aeruginosa*, and *Staphylococcus* spp. as “harmonized method (IP, BP, EP, and USP)” or “harmonized pharmacopeial method (EP, BP, USP, and IP)”. Please explain what this refers to.

The harmonized pharmaceutical method refers to the method of analysis adopted for “2.6.12 Microbiological examination of non-sterile products: Microbiological enumeration test” and “2.6.13 Microbiological examination of non-sterile products: Test for specified microorganisms,”

ref European Pharmacopoeia 8.0 chapter 5.8." These methods are adopted by the European Pharmacopoeia (EP), the Japanese Pharmacopoeia (JP), and the U.S. Pharmacopoeia (USP). As the International Pharmacopoeia (IP) method is same as USP, it is considered as a part of the harmonized method. See Attachment I.

9. The notifier states that the method used to detect total coliforms is "FDA Bacteriological Analytical Manual" (page 18). For the administrative record, please provide the chapter number from the FDA Bacteriological Analytical Manual used for this referenced method.

The reference is "Section G: Solid medium method- Coliforms" under Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria FDA. See Attachment II.

10. Please provide the complete citation(s) for the USP method(s) used for the batch analyses for arsenic, cadmium, mercury, and lead.

Heavy metal analysis is performed as prescribed in USP chapter 233 *Elemental Impurities – Procedures* using Procedure 1: ICP OES. See Attachment III.

11. Please confirm if the batch analyses were conducted on consecutive or non-consecutive batches. If the analyses were conducted on consecutive batches, please provide batch analyses for 3 non-consecutive batches.

The three batches reported in GRN 949 were not non-consecutive. Analyses of three non-consecutive batches are provided in Attachment IV.

12. The results of the batch analyses for arsenic, cadmium, mercury, and lead were listed as "complies" (page 19). Therefore, it is not possible to determine if the specifications for these heavy metals are appropriate based on the results of the batch analyses. Please provide the analytical results for the batch analyses for arsenic, cadmium, mercury, and lead. In addition, please review the specification limits based on these batch analyses and ensure that the specifications are consistent with the analytical results and that the exposure to heavy metals from the intended use of the ingredient is as low as possible.

The analytical results for three non-consecutive batches provided in Attachment IV include the information requested. Additionally, we agree with FDA's suggestion that the heavy-metal specifications be set to ensure that exposure to heavy metals is as low as possible, and we have reduced the specifications to 0.5 mg/kg for all four heavy metals.

13. Please state whether the fermentation process is conducted in a contained, sterile environment.

The fermentation processed is conducted in a contained and sterile environment in a closed vessel.

14. References to "*Salmonella typhimurium*" on page 28 should read *Salmonella* Typhimurium. Please make a statement that corrects this reference.

The citation to tester strains of *Salmonella typhimurium* on page 28 is correct.

I trust these responses to your questions are satisfactory. If you have additional questions, or require further clarification on these, please contact me.

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President

24 pages have been removed in accordance with copyright laws. The removed reference citations are:

- European Pharmacopoeia, 5.8 Pharmacopoeial Harmonisation, 01/2014:508000, 6 pages
- Section G: Solid medium method- Coliforms" under Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria FDA, 18 pages
- U.S. Pharmacopeia, chapter 233 *Elemental Impurities – Procedures* using Procedure 1: ICP OES, 2 pages

Table 1. Analysis of Compositional Variability of *B. coagulans* LBSC

Parameter	Specification	Batch		
		121853	121857	121861
B. coagulans viable spore count	Not less than 150 billion viable spore counts/g	170 billion viable spore count/g	172 billion viable spore count/g	168 billion viable spore count/g
Description	Light brown to brown colored powder	Light brown colored powder	Light brown colored powder	Light brown colored powder
Microscopy/ Identity	Gram positive rods with terminal spores	Complies	Complies	Complies
Sieve test	100% pass through 40 mesh.	Complies	Complies	Complies
Moisture/Loss on drying (%)	Not more than 7.0%	5.20	5.10	5.40
Lactic acid producing capacity	Not less than 10 ml of 0.05 N NaOH consumed	16.1 ml of 0.05N NaOH Consumed	16.3 ml of 0.05N NaOH Consumed	16.2 ml of 0.05N NaOH Consumed
Heavy Metal Analysis				
Arsenic	Not more than 0.5 ppm	< 0.25	< 0.25	< 0.25
Cadmium	Not more than 0.5 ppm	< 0.10	< 0.10	< 0.10
Lead	Not more than 0.5 ppm	< 0.25	< 0.25	< 0.25
Mercury	Not more than 0.5 ppm	< 0.10	< 0.10	< 0.10
Microbial Analysis				
Total Bacterial Count	Not more than 0.2 million cfu/g	12000 cfu/g	13000 cfu/g	10000 cfu/g
Total yeast & mold count	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
Total Coliform	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
E. coli	Absent in 10g	Complies	Complies	Complies
Salmonella	Absent in 10g	Complies	Complies	Complies
P. aeruginosa	Absent in 1g	Complies	Complies	Complies
Staphylococci	Absent in 1g	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25g	Complies	Complies	Complies